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Development of rapid diagnostic tests and screening culture media by the NARA for detection of multidrug resistance

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At the NARA, rapid diagnostic tests for detecting several antibiotic resistance traits in Gram negative have been developed during the latter few years. They target nosocomial and community-acquired *Enterobacterales* (*Escherichia coli, Klebsiella pneumoniae, Enterobacter* spp…) and the hospital-acquired pathogens *Pseudomonas aeruginosa* and *Acinetobacter baumannii.* Among the clinically-relevant antibiotic resistance traits, (i) extended-spectrum ß-lactamases (ESBLs) confer resistance to all β-lactams with the exception of carbapenems, (ii) carbapanemases confer virtually resistance to all β-lactams including carbapenems, and (iii) and 16S rRNA methylases confer resistance to all clinically-used aminoglycosides. In addition, the spread of polymyxin resistance determinants may be another step towards pandrug resistance in Gram-negative bacteria. It is now widely accept that use of rapid tests may decrease of morbidity and mortality related to antibiotic resistant bacteria [Burnhams et al., 2017; Paul et al., 2010; Schwaber et al., 2007]

We have developed rapid diagnostics with the goal to propose sensitive and specific techniques with a turn-around-time (TAT) of a maximum of 3-4 h. The developed tests are based either on biochemistry methods, on rapid culture approaches, and also on genetic techniques. All those tests are used routinely since years at the NARA. Screening culture media for detection of multidrug resistance have been also developed. In addition, a screening culture medium has been developed for detection of linezolid-resistant Gram positives.

1- Rapid Diagnostic Tests

1.1 Rapid detection of ESBL producers in *Enterobacterales*

Extended-spectrum-β-lactamase (ESBL)-producing *Enterobacterales* (ESBL-E) represent a worldwide clinical issue, especially given their association with multidrug resistance patterns, severity of illness, poor outcomes, and growing number in the community [Zahar et al., 2009]. ESBL-producing *Enterobacterales* (ESBL-E) constitute a global burden and a major threat to public health. The production of ESBLs precludes the use of broad-spectrum cephalosporins, making carbapenems the drug of choice for treating septicemia due to ESBL producers. Thus, the increased prevalence of those organisms has stimulated the empiric use of carbapenems as therapy when ESBL-E are suspected, favoring selection of carbapenem-resistant *Enterobacterales*. Rapid detection of ESBL-E therefore constitutes a challenge for clinical microbiologists to prevent delaying efficient antibiotic therapy that might worsen the survival of the most severely-ill patients.

Traditional techniques for detecting ESBL-E include phenotypic tests and molecular tests (to detect the genes encoding such enzymes) and have been widely developed by others.
The rapid ESBL NDP test that was first developed as a home-made test for detecting the hydrolysis of cefotaxime (and any other broad-spectrum cephalosporins) in less than 30 min by using a pH indicator (phenol red) [Nordmann et al., 2015, Decousser et al., 2017]. In comparison to a negative control well without antibiotic, the reactive well containing cefotaxime and the pH indicator experiences a color change from red to yellow if ESBL produces some carboxyl-acid groups resulting from cefotaxime hydrolysis. The same reaction occurs in the presence of a penicillinase inhibitor, namely tazobactam, which inhibits the hydrolysis reaction, thereby helping to identify the ESBL nature of the ß-lactamase (Figure 1). The performance of this home-made test has been evaluated with either cultured bacteria (sensitivity, 92.6%; specificity, 100%) or directly from urine (sensitivity, 98%; specificity, 99.8%) or positive blood culture samples (sensitivity, 100%; specificity, 100%). Its sensitivity is excellent, particularly for detecting CTX-M producers (100%) [Nordmann et al .2015; Dortet et al., 2015]. This test identifies ESBLs from bacterial culture, blood and urines [Dortet et al., 2014; Dortet et al. 2015; Poirel et al., 2016].

The ESBL NP test (LiofilChem, Italy) is an updated and industrialized version of the ESBL NDP test (Figure 2) [Blanc et al., 2021; Boattini et al., 2022; Demord et al., 2021]. Its excellent performance for detecting ESBL activity has been validated by using positive blood cultures (hence contributing to an optimal early antibiotic therapy) and bacterial cultures resulting from screening of carriers of ESBL producers (hence contributing to improve the infection control practices).

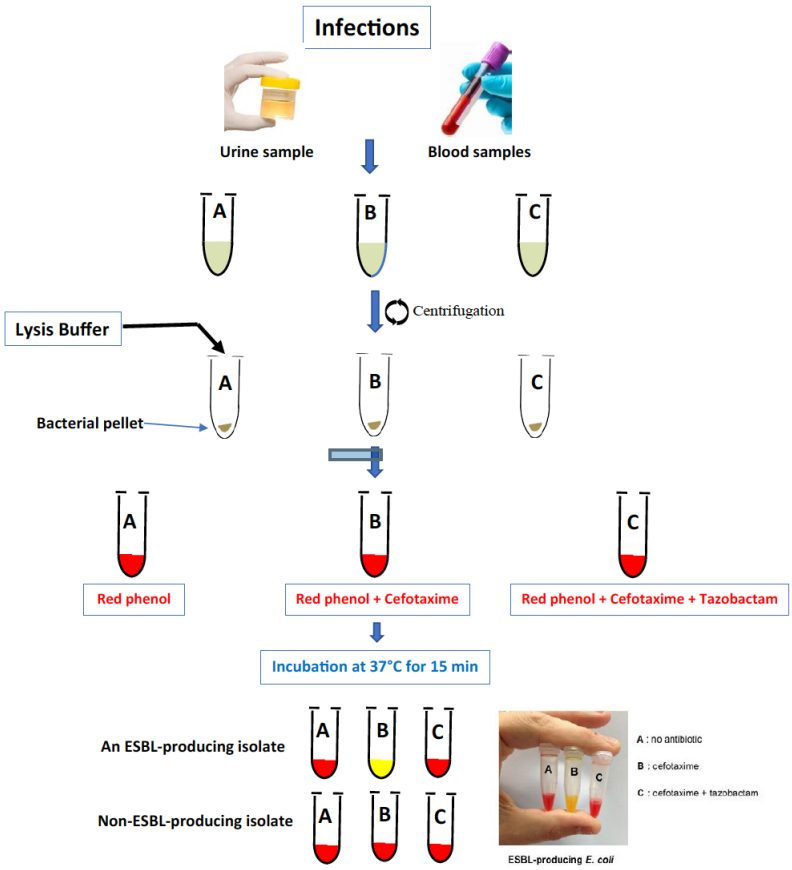


Figure 1. Strategy for detecting ESBL producers from clinical samples (urines, blood samples) using the Rapid ESBL test

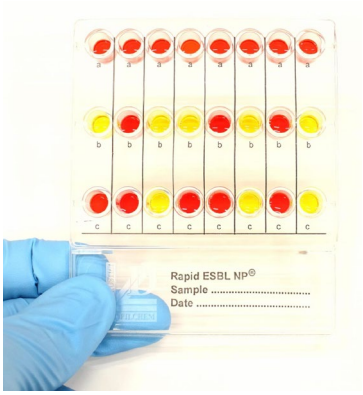


Figure 2. Rapid ESBL NP test (LiofilChem, Italy)

1.2 Rapid detection of carbapenemase producers

Acquired resistance to carbapenems https://www.sciencedirect.com/topics/medicine-and-dentistry/carbapenem is a global problem enhanced by plasmid-mediated spread of class A (KPC, GES), class B (IMP, VIM, NDM) and class D (OXA-48-like) carbapenem-hydrolyzing β-lactamases among multiple species of *Enterobacterales*, including *Escherichia coli*, *Klebsiella*, *Enterobacter*, and *Citrobacter* species [Nordmann et al., 2019]. In Switzerland th most frequent carbapenemases in *Enterobacterales* are of the OXA-48 types. There is an increasing number of hospital outbreaks due to those bacteria which are also resistant to many other families of antibiotics. These outbreaks are difficult to control and overlap with spread to and within the community. Whereas KPC producers remain mostly hospital-acquired *K. pneumoniae*, spread of VIM, NDM and OXA-48-like producers is now observed in hospital and community-acquired infections since those latter carbapenemase genes are located in a variety of enterobacterial species such as in *E. coli*. Although globalization of carbapenemase producers in ongoing, endemicity of KPC producers is known worldwide in particular in the USA, Italy, Greece, Portugal, and India. NDM producers in North Africa, Middle East, Eastern Europe, Asia, and OXA-48-like producers in North Africa, Africa, Middle East, Africa and India.

In Switzerland, we are identifying an increasing number of carbapenemase producers not only in *Enterobacterales* but also in *Pseudomonas aeruginosa* and in *Acinetobacter baumannii*. The carbapenemases identified in *P. aeruginosa* are mostly of VIM type (VIM-2), GES type (GES-5) in particular, and more rarely of NDM, IMP and exceptionally of the KPC type. By contrast the carbapenemases in *A. baumannii* are mostly specific to that species, i.e. class D enzymes being OXA-23, OXA-40 and OXA-58. More rarely carbapenemases of the NDM type are identified in that species.

1.2.1 Rapid Carba NP test

A biochemical detection of carbapenemases has been developed based on the rapid detection of imipenem hydrolysis [Rapid Carba NP test] (Figure 3) [CLSI/EUCAST guidelines, 2019]. The principle of this test is based on the detection of the acidification of a reaction medium when the ß-lactam ring of the carbapenem is open by the carbapenemase activity [Nordmann et al., 2012]. The modification of the medium pH is revealed by a colour shift in the pH indicator, namely the red phenol that turns red to yellow upon acidification.

Results are obtained within 30 min-2 h with good specificity and sensitivity. It detects any kind of carbapenemase activity. A few OXA-48-like strains produce their carbapenemases at low level, challenging the detection. Use of an adequate inoculum (1 öse of 10 µl) is critical to detect those low-level carbapenemase producers. This test is adequate for detection of carbapenemase activity for *Enterobacterales* and *P. aeruginosa* [Dortet et al., 2012; Dortet et al., 2013; Dortet et al., 2014]. A commercialized version of this test is available (Rapidec Carba NP test, bioMérieux [Figure 4]) [Mancini et al., 2017; Poirel et al., 2015]. For detection of any carbapenemase activity in *A. baumannii*, extraction reagent, lysis buffer and inoculum have to modified accordingly, corresponding to the so-called CarbAcineto NP test derived from the Rapid Carba NP test [Dortet et al., 2014].

The sensitivity of those rapid tests can be negatively influenced by several culture media such as the Drigalski or MacConkey agar plates, since coloured pigments may interfere with the reading of the test [Garcia-Quintanilla et al., 2018].

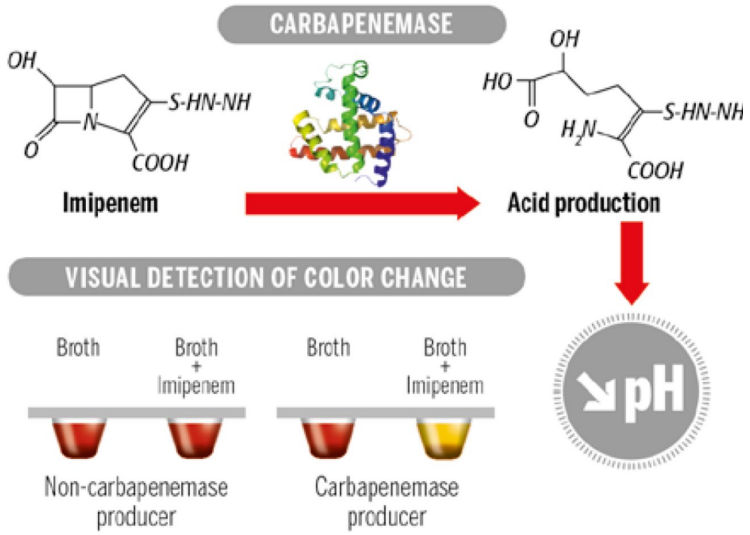
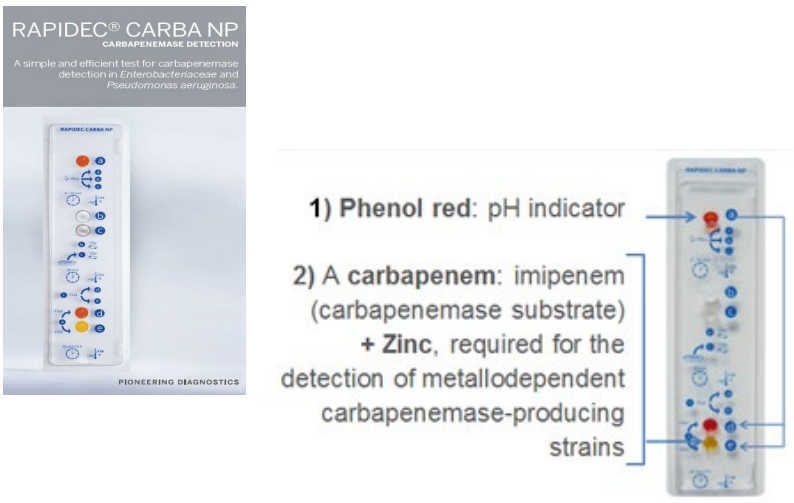
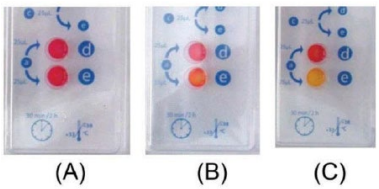


Figure 3. Principle of the Rapid Carba NP test





(A) Negative result (red) (B) Weak result (orange) (C) Strong result (yellow)

Figure 4. The Rapidec Carba NP test

1.2.2 NitroSpeed Carba NP test

Since the early development of the Rapid Carba NP test, several novel combinations of ß-lactams / ß-lactamase inhibitors have been marketed. They are ceftazidime.avibactam, meropenem.vaborbactam and imipenem.relebactam. None of those combinations of molecules is active against producers of metallo-carbapenemases. Avibactam is active against carbapenemases of Ambler class A (KPC) and Ambler class D (OXA-48 like) whereas vaborbactam and relebactam are active only against KPC producers. Therefore, there was a need for rapid identification of the carbapenemase types, in order to contribute to use the most adequate treatment options.

The Nitro-SpeedCarba NP test has been developed to reach this goal [Nordmann et al., 2020]; (Figure 5). This is a chromogenic test based on detection of nitrocefin hydrolysis (visually detected color change from yellow to red). After an extraction step with a lysis buffer of a culture of an enterobacteral isolate, the protein extract is submitted to a carbapenem (ertapenem)-containing solution. If the protein extract contains a carbapenemase, ertapenem is hydrolyzed as well as nitrocefin. If the protein extract contains either no ß-lactamase of a non-carbapenemase ß-lactamase, ertapenem enters the active site of the ß-lactamase and blocks its action. The nitrocefin which is added in a second step is consequently not hydrolyzed. In order to identify the type of carbapenemase produced, a further step was added prior to ertapenem adding, which is inhibition by avibactam (inhibition of class A and D), by vaborbactam (inhibition of class A) or by dipicolonic acid (inhibition of class B). Sensitivity and specificity of the test is 100% and 97%, respectively. Its TAT is less than 30 min. In most of the cases, results of the test are obtained in 1 min. The OXA-48-like producing strains were very well detected within the 20 min period of the test. A specificity of the test is not 100% since a few *Klebsiella oxytoca* strains that produce a naturally-occurring ß-lactamase of very weak although detectable carbapenemase activity give false-positive results. Overall the Nitro-Speed Carba NP test possesses and excellent sensitivity for detection of carbapenemase activity. In addition, the Nitro-Speed Carba NP test allows identification of the type of carbapenemases, namely class A, B or D based on the differential inhibitory activity of relebactam, avibactam, and dipicolinic acid. This test is also adapted also for the identification of carbapenemases in *P. aeruginosa* but not for those of *A. baumannii* [Sadek et al., 2021]. It is available so far only as an home-made version.

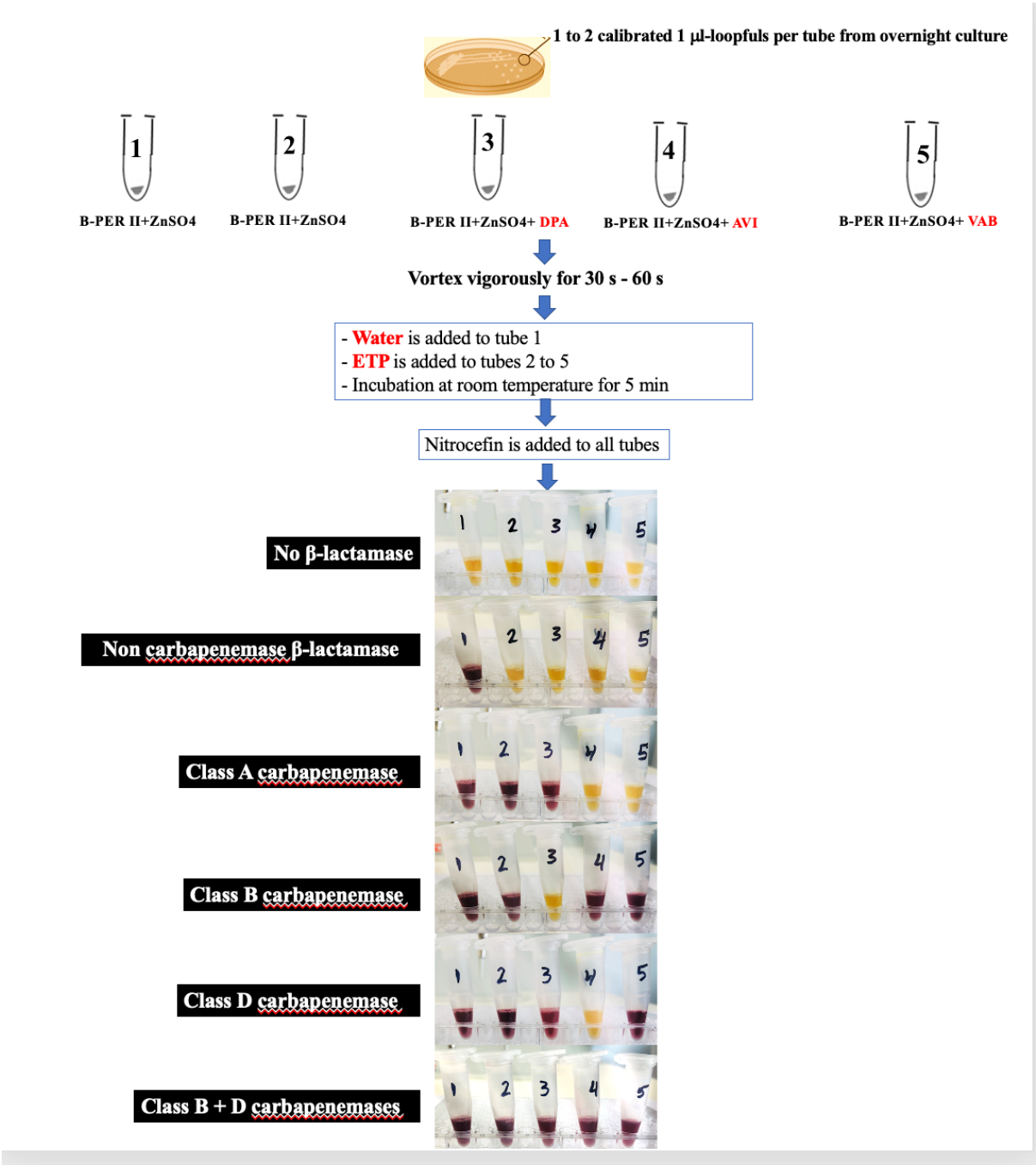


Figure 5. The NitroSpeed-Carba NP test. DPA, dipicolonic acid; AVI, avibactam, VAB, vaborbactam; ETP, ertapenem. B PER II is the lysis buffer.

1.3 Rapid detection of carbapenem resistance

1.3.1 Rapid detection of carbapenem resistance in *A. baumannii*

The rapid ResaImipenem/*Acinetobacter* NP test was developed for the identification of carbapenem resistance among *A. baumannii* [Nordmann et al., 2021], (Figure 6).

The carbapenem resistance trait is systematically associated with carbapenemase production in that species. The principle of this test is based on the reduction of resazurin (a viability colorant) by metabolically active bacterial cells, hence detecting bacterial growth, in the presence of a defined concentration of imipenem chosen to be slightly above that defining imipenem resistance (6 mg/ml). Bacterial growth is visually detected by a color change from blue (resazurin) to purple or pink (resorufin product). A total of 110 *A. baumannii i*solates, among which 61 were imipenem resistant, were used to evaluate the test performance. The sensitivity and specificity of the test were found to be 100%, in comparison with broth microdilution taken as the reference standard method. The rapid ResaImipenem/*Acinetobacter* NP test is highly specific and sensitive and is easy to implement in routine microbiology laboratories, and results are obtained within 3-4 h. It does not require any additional equipment.

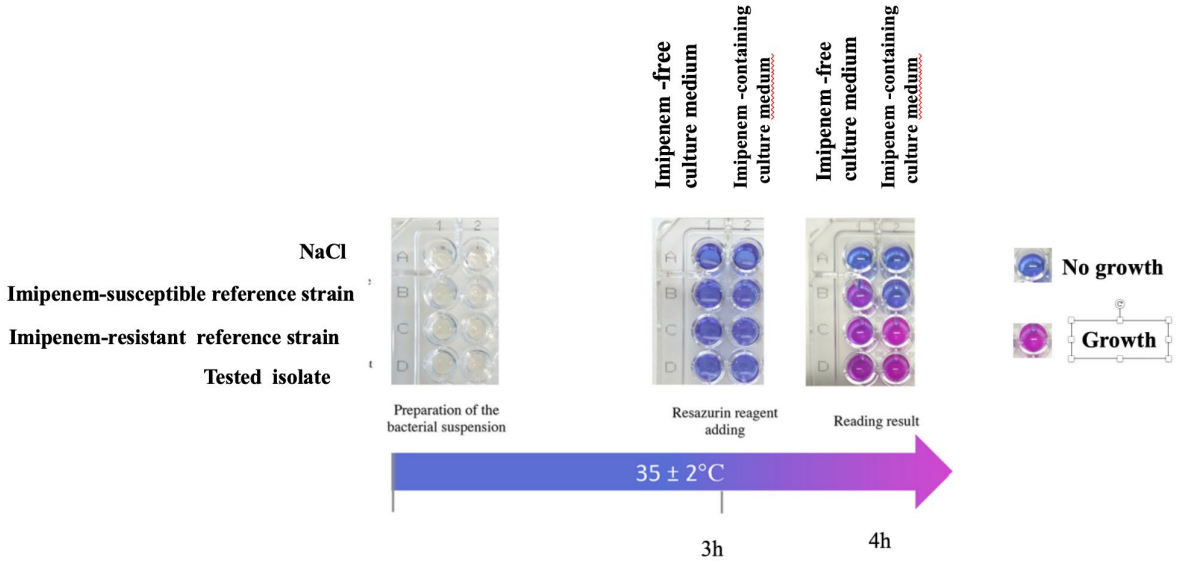


Figure 6. The Rapid ResaImipenem *Acinetobacter* NP test

1.4 Rapid detection of polymyxin resistance

The spread of carbapenem resistance strains in *Enterobacterales*, *P. aeruginosa* and *A. baumannii* that is associated with a multidrug resistance pattern has contributed to a renewal interest of clinical use of polymyxins. Those antibiotics (colistin, polymyxin B) are often considered as last report therapeutics in association with other antibiotic groups for treating infections due to those multidrug-resistant bacteria. Polymyxins are polycationic and bactericidal drugs that target the lipid A moiety of the lipopolysaccharide (LPS), moving its cationic charges and subsequently leading to cell wall lysis and bacterial death. The increasing use of colistin in animals since years but also nowadays in humans has led to emerging resistance to polymyxins (cross resistance between colistin and polymyxin B). Until recently, all known resistance mechanisms involved upregulation or dysregulation of chromosomal genes encoding proteins mainly related to regulatory systems of the LPS biosynthesis, such as the PmrAB and PhoPQ two-components systems in *Enterobacterales* [Poirel et al., 2017].

In November 2015, the first plasmid-mediated colistin resistance gene, namely *mcr-1*, that encodes a phosphoethanolamine transferase, was identified in enterobacterial isolates [Poirel et al., 2017]. This enzyme transfers a phosphoethanolamine group to the LPS moiety leading to a modified LPS structure preventing binding with polymyxins. There after other *mcr*-like genes, namely *mcr-2* to *mcr-10* that have then been identified [Poirel et al., 2017]. They were reported worldwide mostly from animal isolates in accordance with the high selection pressure with polymyxins in veterinary medicine. Although they are reported in clinical strains from humans in Switzerland [Nordmann et al., 2016: Mueller et al. 2019], their spread among human pathogens in this country remains rare [Bourrel et al., 2019; Poirel et al., 2017].

Resistance to colistin in human isolates of *Enterobacterales* is mostly related to non-MCR and chromosome-encoded mechanisms [Poirel et al., 2017]. We are currently observing at a worldwide scale, the spread of colistin-resistant strains (with a chromosome-encoded mechanism of resistance) among carbapenemase producers (KPC, OXA-48, NDM). Such strains have been identified in Switzerland resulting from an importation process (mostly from Italy and Greece) but also from direct selection in Switzerland following the treatment of patients with colistin [Poirel et al., 2017].

A particular concern is that the majority of the MCR producers may be difficult to detect since this resistance determinant confers low MIC values (around 4 mg/L) while chromosomally-encoded resistance mechanisms confer much higher levels of resistance (usually > 64 mg/L).
So far resistance to polymyxins in *P. aeruginosa* and *A. baumannii* is only chromosome-encoded with the exception of very few plasmid-encoded MCR-4 in that latter species [Poirel et al., 2017]. They are mostly related to changes in LPS structure.

The reference technique for determining susceptibility to polymyxins in Gram negatives remains the determination of MICs in liquid medium [Poirel et al., 2017; Jayol et al., 2017; Jayol et al., 2018]. It requires at least 18 h. Therefore, rapid techniques for identification of the polymyxin susceptibility/resistance have been developed at the NARA. They are based on a rapid culture with a given concentration of colistin that inhibits the growth of susceptible strains.

1.4.1 Rapid detection of polymyxin resistance in *Enterobacterales*

The Rapid Polymyxin NP test is based on a change of pH according to glucose metabolism related to bacterial growth (Figure 7). Detection of colistin resistance is made according to growth in presence of a given concentration of colistin (3 mg/L). The test has been validated using bacterial cultures and positive blood cultures [Nordmann et al., 2016; Jayol et al., 2016; Jayol et al., 2018; Poire et al., 2017]. It offers the important advantage of a TAT for results of 2-3 h. It detects any type of polymyxin resistance determinant (chromosomal and plasmid-encoded). Compared to the broth microdilution susceptibility method, there is excellent agreement for detecting polymyxin resistance including that related to MCR enzymes. The Rapid Polymyxin NP test has also a good sensitivity to detect naturally-occurring polymyxin resistance mechanisms such as those observed in *Hafnia alvei*. However, it fails to detect several colistin-resistant *Enterobacter* sp. isolates probably due to the frequent colistin heteroresistance pattern observed in that species. This test is now commercialized available under the trade name of Rapid Polymyxin NP test (ELITech Group, France) that provides the opportunity of testing up to 10 samples in a row (Figure 7). Its sensitivity and specificity are 99.3% and 94/9%, respectively.

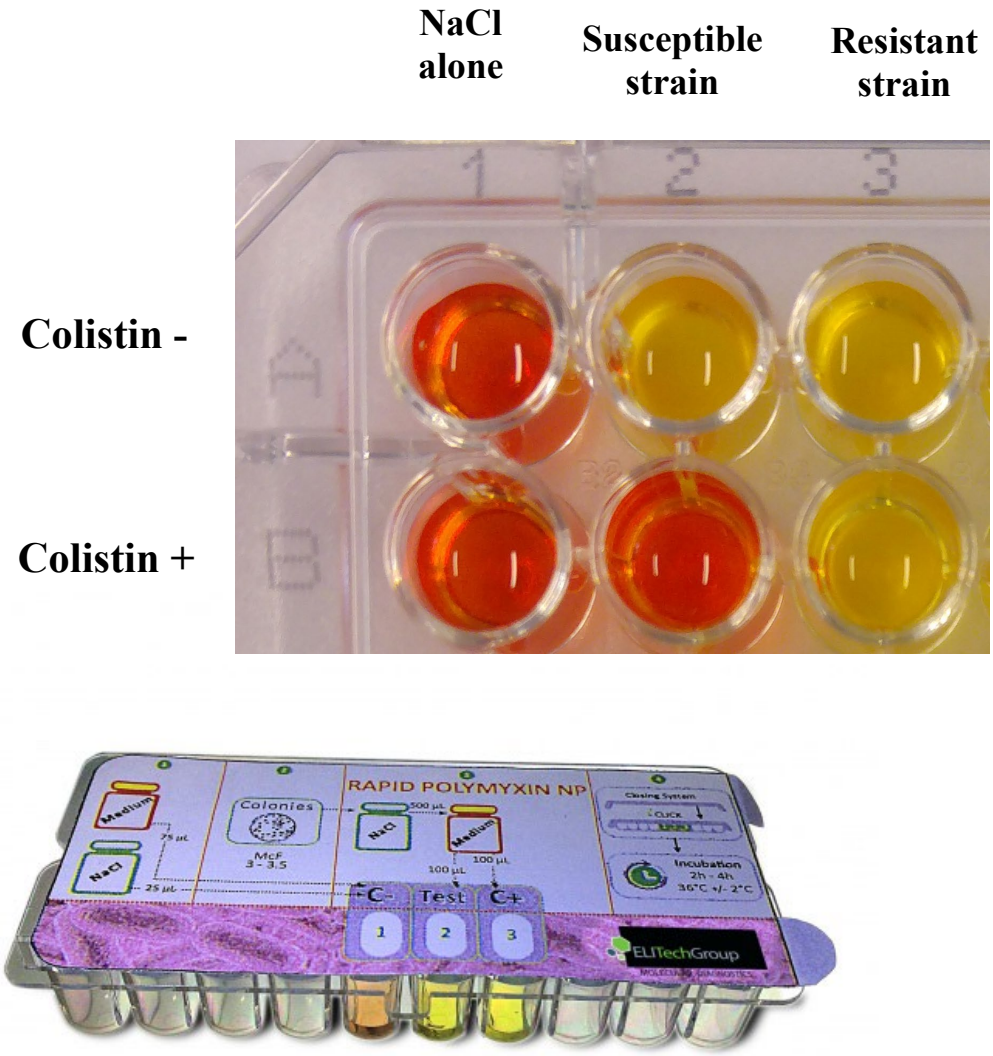


Figure 7. Rapid Polymyxin NP for Enterobacterales and its industrialized version

1.4.2 Rapid detection of polymyxin resistance in *A. baumannii*

Multidrug resistance in *A. baumannii* is now observed in Switzerland mostly in strains isolated from patients having been primarily hospitalized abroad. The Rapid Polymyxin NP test we had developed for rapid identification of polymyxin resistance in *Enterobacterales* does not give satisfactory results with *A. baumannii* strains and is therefore not recommended for that species. Actually, this test is based on detection of glucose metabolism (color change of a pH indicator) related to bacterial growth in the presence of a determined concentration of colistin, which explains the failures observed with the non-fermenter *A. baumannii* species [Lescat et al. 2019], (Figure 8). We have therefore developed a new assay based on the utilization of resazurin (7-hydroxy- 3H-phenoxazin-3-one-10-oxide), a viability colorant, also referred to as AlamarBlue and PrestoBlue. Its principle is based on the fact that metabolically active cells reduce blue resazurin to the pink product resorufin. This reduction is proportional to the number of metabolically active cells. Therefore, we have developed a test, based on a comparison of bacterial viabilities, after growth in medium with or without a defined concentration of colistin (3.75 mg/L). The principle of this test is based on the visual detection of the reduction of the resazurin reagent, a viability colorant, as observed by its color change. The Rapid ResaPolymyxin *Acinetobacter*/ NP test is cost effective, easy to perform, highly sensitive and specific, and can be completed in 3- 4 hours. It has been industrialized under the trade name of Rapid Resa Poly Acineto NP test (LiofilChem, Italy) making possible to test several samples at the same time (Figure 9) [Bouvier et al., 2021].

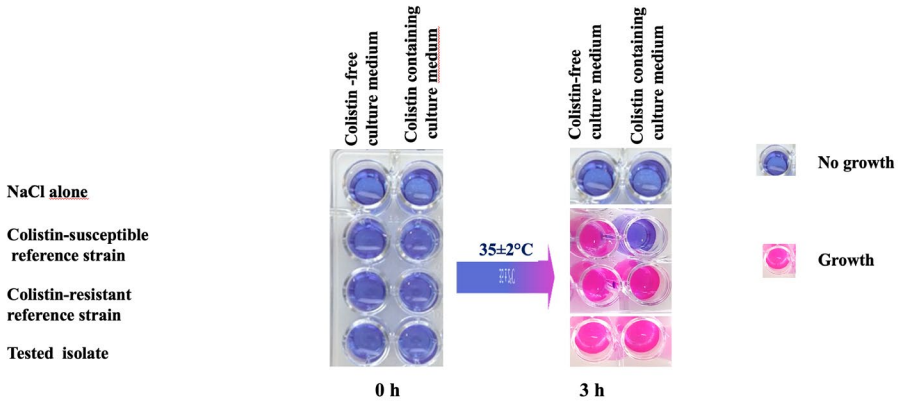


Figure 8. The Rapid Resa Polymyxin Acinetobacter NP test

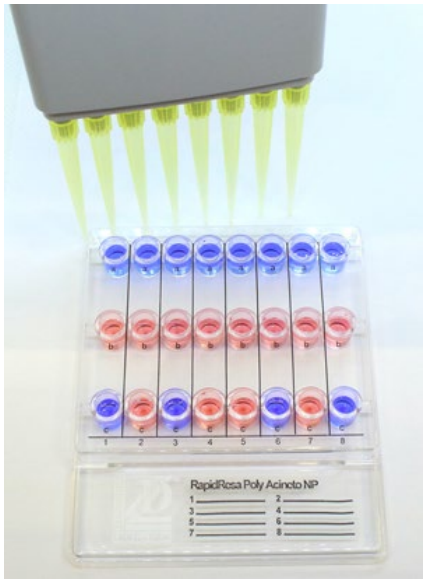


Figure 9. Industrialized version of the RapidResa Poly Acinetobacter NP test

1.4.3 Rapid detection of polymyxin resistance in *P. aeruginosa*

Having considered that growth of *P. aeruginosa* is associated with production of basic metabolites as opposed to growth of Enterobacterales that lead to production of acidic metabolites, we have therefore used this property for developing a novel specific test (Rapid Polymyxin/*Pseudomonas* NP test) [Sadek et al., 2020]. Formation of basic metabolites is visually detected by yellow to purple/violet color change of the bromocresol purple pH indicator in the presence or absence of a given concentration of polymyxin (4 mg/L) [Figure 10]. The sensitivity and specificity of the test were found to be 100% and 96.2%, respectively, by comparison with broth microdilution reference method. The Rapid Polymyxin/*Pseudomonas* NP test is easy-to-perform, specific and sensitive, and allows rapid visual observation of results without the requirement of any special reading equipment, results being obtained within 3 h. This test is so far available as an home-made version only.

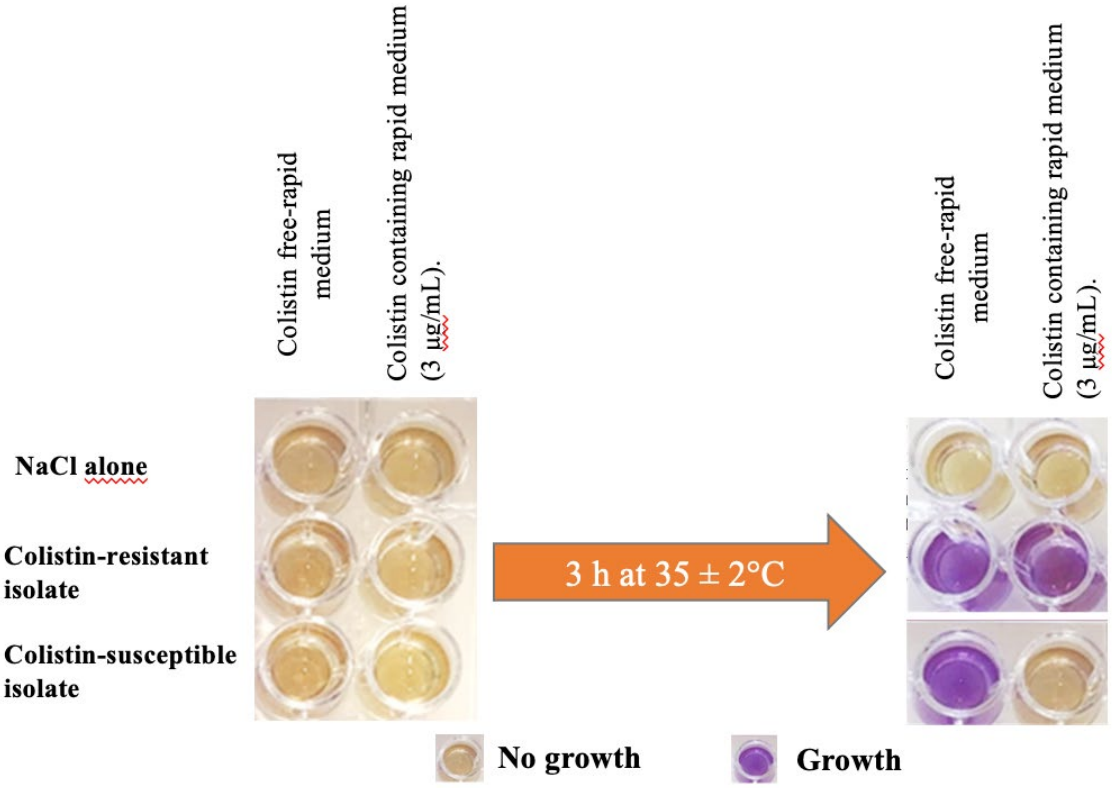


Figure 10. The Rapid Polymyxin/Pseudomonas NP test

1.4.4 Multiplex PCR for identification of MCR-encoding genes in *Enterobacterales*

A rapid (total time <2 h) and reliable multiplex polymerase chain reaction for screening of *mcr-1 to mcr-5* genes conferring resistance to colistin has been developed [Bontron et al., 2016; Lescat et al., 2018]. Real-time PCR techniques have been also developed for detection of *mcr-1*/*mcr-2* genes with excellent sensibility and specificity. An updated version of those techniques has been developed making possible the detection of *mcr-1 to mcr-10* genes. It is available upon request. Those techniques can be easy adapted in laboratory possessing a PCR machine.

1.5 Rapid detection of fosfomycin resistance in *E. coli*

Fosfomycin has been approved for the treatment by single dose oral therapy of uncomplicated urinary tract infections and now proposed as first line and empirical therapy of uncomplicated urinary tract infections. However, susceptibility to fosfomycin is variable and several plasmid-mediated *fosA*-like genes and chromosome-mediated resistance mechanisms have been identified, either modifying the structure of fosfomycin or interacting with its transport or its target, respectively [Cattoir et al., 2018, Nordmann et al., 2022].

Based on the principle of the Rapid Polymyxin NP test in *Enterobacterales*, we have developed a rapid fosfomycin test for *E. coli* [Nordmann et al., 2029]. *E. coli* is a bacterial species that does not possess any intrinsic *fos*-like resistance gene. The test is based on detection of glucose metabolism (visual detection of a color change of a pH indicator) related to bacterial growth in the presence of a determined concentration of fosfomycin (40 mg/L).

Its sensitivity and specificity are 100% and 98.7%, respectively, whatever the resistance mechanism to fosfomycin is (FosA or non-plasmid mediated resistance mechanisms). Its TAT for results is 1 h 30. However, it does not work with strains belonging to other enterobacterial species (K. pneumoniae, Enterobacter sp, Serrratia sp.) since expression of the naturally-occurring FosA determinants naturally present in those species interfere with the specificity of the results. This test is so far only available in its home-made version.

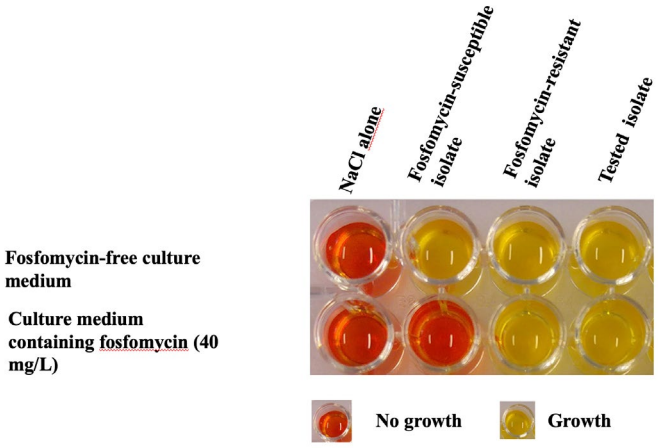


Figure 11. Rapid fosfomycin NP test

1.6 Rapid detection of pan-resistance to aminoglycosides

The most prevalent mechanisms of resistance to aminoglycoside (AG) in *Enterobacterales* are enzymes that modify the chemical structure of aminoglycosides and can be either nucleotidyltransferases, phosphotransferases, or acetyltransferases [Doi et al., 2016]. The genetic determinants coding for those aminoglycoside-modifying enzymes are often present in various combinations in a given strain and are typically found on plasmids. They do not confer cross-resistance to all AG molecules.

However, plasmid-mediated 16S rRNA methyltransferases conferring a high level of resistance to multiple AG has also been reported, and they are identified at a high frequency, especially among producers of NDM-like carbapenemases [Doi et al., 2016]. Enzymes encoded by these genes methylate the aminoglycoside tRNA recognition site (A-site) of the 16S rRNA, which is the intracellular target of the AG. The 16S rRNA methylases identified in *Enterobacterales* are ArmA, RmtB to RmtF, and NpmA, with ArmA being the most frequently identified methylase. Those methylases exhibit a significant amino acid heterogeneity between each other, sharing similar amino acid motifs in their active sites. They confer resistance to almost all AG (amikacin, gentamicin, tobramycin, and kanamycin, but not neomycin), whereas NpmA confers additional resistance traits to neomycin (given as a topical agent) and to apramycin (not used for humans).

In an area of paucity of novel molecules, rapidly identifying multiple resistance to AG by a simple test may be useful for implementing antibiotic stewardship and containment of those multidrug-resistant bacteria.

The rapid aminoglycoside NP (Nordmann/Poirel) test was developed to rapidly identify multiple aminoglycoside (AG) resistance in *Enterobacterales* [Nordmann et al., 2017]. It is based on the detection of the glucose metabolism related to enterobacterial growth in the presence of a defined concentration of amikacin plus gentamicin (Figure 11). Formation of acid metabolites was evidenced by a color change (orange to yellow) of the red phenol pH indicator. The rapid aminoglycoside NP test was evaluated by using bacterial colonies of 18 AG-resistant isolates producing 16S rRNA methylases, 20 AG-resistant isolates expressing AG-modifying enzymes (acetyl-, adenyl-, and phosphotransferases), and 10 isolates susceptible to AG. Its sensitivity and specificity were 100% and 97%, respectively, compared to the broth dilution method, which was taken as the gold standard for determining aminoglycoside resistance. The test is inexpensive, rapid (2 h), and implementable worldwide. It is so far available only in its home-made version.

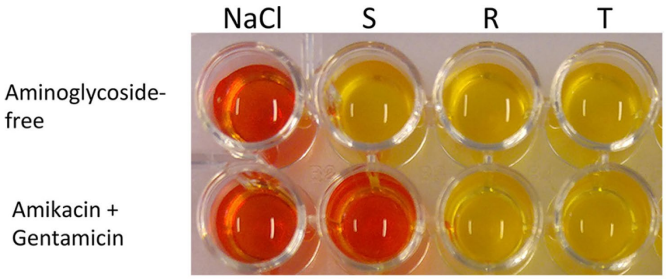


Figure 11. The Rapid Aminoglycoside NP test. The Rapid Aminoglycoside NP test was performed with a reference susceptible isolate (S) in the second column and with a reference 16S rRNA methylase *K. pneumoniae* isolate (ArmA) (R) in the third column in a reaction medium without (upper) and with (lower) the AG mix (amikacin plus gentamicin). Non-inoculated wells are shown in the first column (NaCl- containing wells). The tested isolate (T), in the fourth column, grew in the presence of the mix of aminoglycosides and therefore presented resistance to multiple aminoglycosides. The picture was taken after 2 h of incubation.

2- Screening media for multidrug resistance

A series of screening culture medium has been developed for detecting emerging antibiotic resistant bacteria mostly for controlling outbreaks in a nosocomial context. The availability of those culture media is important at the bedside for screening carriers at the stage of colonization before infections occur. Use of chromogenic molecules in most of those culture media contribute to the identification of bacterial species. The specimens of choice for those screenings remain stools and rectal swabs. Those screening media have been validated at least with cultured bacteria and spiked stools. Such screening culture media as developed by our group correspond to totally novel screening media (not being variants of existing media). In many instances, the home-made screening media have been industrialized and therefore become easy available for any clinical and hygiene laboratory.

2.1 Resistance to carbapenems in *Enterobacterales*

A Drigalski agar-based culture medium containing an ertapenem (0.25 mg/L), cloxacillin (250 mg/L), and zinc sulfate (namely the Supercarba medium) was tested for screening carbapenemase-producing members of the family *Enterobacterales* [Nordmann et al, 2012] OXA-48 (n=44), NDM (n=25), VIM, IMP (n=27), and KPC producers (n=18) were detected with a low detection limit. The overall sensitivity (95.6%) of that selective medium was high. The Supercarba medium provides a significant improvement for detection of the most common types of carbapenemase producers as well as non-carbapenemase related carbapenem resistance in *Enterobacterales*. It is not aimed to detect carbapenem resistance in *A. baumannii* or *P. aeruginosa.* This medium has been extensively validated and industrialized by adding chromogenic molecules under the trade name CHROMAgar SuperCarba (CHROMAgar, France) (Figure 12) [Girlich et al., 2013; Garcia-Quintanilla et al., 2018].

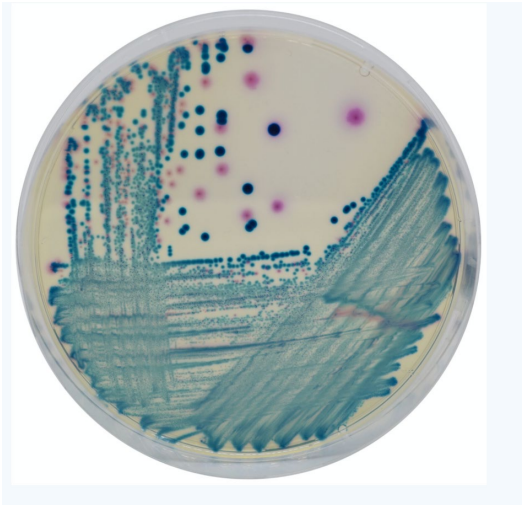


Figure 12. The CHROMagar mSuperCARBAmedium

2.2 Resistance to carbapenems in *Pseudomonas aeruginosa*

A selective culture medium for screening carbapenem resistance in *Pseudomonas* spp has been established recently. Carbapenem-resistant *Pseudomonas* sp. are frequent sources of nosocomial outbreaks in particular within intensive care units. This selective medium contains meropenem (2 mg/L) and a CHROMagar Pseudomonas medium as base (CHROMagar, France [Figure 13] (Nordmann et al., 2021]. It has been evaluated using 29 meropenem-susceptible and 56 meropenem non-susceptible *Pseudomonas*-like clinical isolates, the latter exhibiting a variety of carbapenem-resistance mechanisms. Its sensitivity and specificity of detection were found to be 91% and 100%, respectively. By testing spiked stools, an excellent performance of the medium was also observed for detection of carbapenem-resistant *P. aeruginosa*, with lowest detection limit ranging from 10^0 to 10^2 CFU/ml. This screening medium provides the opportunity to select carbapenem-resistant *Pseudomonas* and *Pseudomonas*-related isolates regardless of their resistance mechanism. Its industrialization is currently in development (LiofilChem, Italy).

Figure 13. The Chromatic SuperCP medium

2.3 Resistance to ceftazidime/avibactam

Among the recently developed agents active against multidrug-resistant Gram-negative pathogens, a novel drug combination has been launched, namely ceftazidime-avibactam (CZA). Avibactam (AVI) is a non-β-lactam–β-lactamase inhibitor that inhibits the activities of Ambler class A, class C, and some class D β-lactamases, including carbapenemases (e.g. KPC, OXA-48). However, acquired resistance to CZA is increasingly reported and is mostly related to amino acid substitutions in the active sites of the respective β-lactamases [Nordmann et al., 2019]. Many studies have identified KPC variants in *K. pneumoniae*, such as KPC-31, KPC-35, KPC-41, and KPC-50, all conferring resistance to CZA. Those KPC variants confer acquired resistance to CZA to the corresponding producers mainly as a consequence of increased rate of hydrolysis of ceftazidime (CAZ), but also due to relatively reduced inhibitory activity of AVI. Most of those variants possess a lower carbapenemase activity and therefore may not be detected by using selective culture media containing carbapenem molecules (Hernandez-Garcia et al. 2022). In addition, resistance to CZA in Gram-negative bacteria may be related to the production of Ambler class B enzymes (metallo-β-lactamases [MBL]), such as NDM, VIM, and IMP, or of several non-OXA-48-like class D β-lactamases, such as OXA-28 or OXA-232, whose hydrolytic activity include CAZ but which are not inhibited by AVI. Furthermore, CZA resistance may be related to overproduction of efflux pumps and/or porin defects. Taking in account the increasing use of the CZA combination and consequently the increasing isolation of CZA-resistant Gram-negative bacteria, we have developed a selective culture medium for screening CZA-resistant isolates among Gram-negative species (*Enterobacterales*, *P. aeruginosa*) (Figure 14).

This SuperCAZ/AVI medium contains CAZ (6 mg/L) and AVI (4 mg/L) and chromogenic molecules (CHROM agar Orientation medium, CHROMagar). It was evaluated using 50 CZA-susceptible and 42 CZA-resistant Gram-negative isolates. Its sensitivity and specificity of detection were 100%. Excellent performance of this medium was also observed by testing spiked stools, with the lower limit of detection ranging from 10^1 to 10^2 CFU/ml. This screening medium provides the opportunity to detect CZA-resistant isolates regardless of their resistance mechanisms. An industrialized version of this medium, Chromatic SuperCAZ-AVI medium is now available (LiofilChem, Italy) [Sadek et al., 2021]

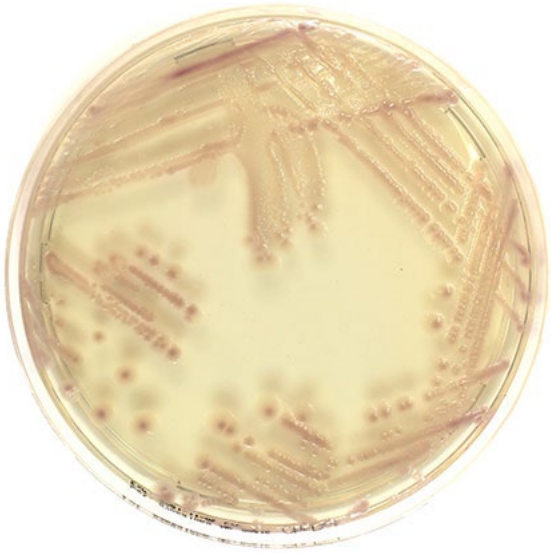


Figure 14. The chromatic SuperCAZ AVI medium

2.4 Resistance to polymyxins

The colistin-containing SuperPolymyxin medium was developed for screening polymyxin-resistant Gram-negative bacteria [Nordmann et al., 2016] (Figure 15). It was the first culture medium aimed to specifically screen for those resistant bacteria. It was specifically formulated to avoid swarming of *Proteus* spp. (naturally resistant to polymyxins) and to prevent contamination by Gram-positive bacteria and fungi. It contains colistin at a concentration of 3.5 mg/L. It was evaluated with 88 polymyxin-susceptible or polymyxin-resistant cultured Gram-negative isolates. Its sensitivity and specificity of detection were near 100%. The SuperPolymyxin medium is the first screening medium that is able to detect intrinsic and acquired polymyxin-resistant bacteria. It has been subsequently extensively evaluated. It is now commercialized under the trade name SuperPolymyxin medium by ELITech Group (France).

Figure 15. The SuperPolymyxin medium

2.5 Resistance to fosfomycin

A selective medium for screening fosfomycin (FOS)-resistant *Enterobacterales* was developed. It contains fosfomycin (16 mg/L), glucose-6-phosphate (25 mg/L) and chromogenic molecules (CHROMagar orientation medium (CHROMagar, France) (Nordmann et al. 2021]. Performances of this medium were first evaluated by using cultures of a collection of 84 enterobacterial clinical strains (42 FOS susceptible and 42 FOS resistant). The SuperFOS medium showed excellent sensitivity and specificity of detection (100%) in those conditions. Then, by testing spiked stool and spiked urine specimens, it revealed excellent performances, with lower limits of identification ranging from 10^1 to 10^2 CFU/ml. This screening medium allows easy and accurate detection of FOS-resistant isolates regardless of their resistance mechanisms. It is only available so far as an home-made version.

2.6 Pan-resistance to aminoglycosides

The SuperAminoglycoside medium containing amikacin plus gentamicin was developed for screening multiple aminoglycoside resistance among Gram-negative bacteria (*Enterobacteriaceae*, *P. aeruginosa*, and *A. baumannii*) [Nordamnn et al., 2018] (Figure 16). It contains a combination of the two aminoglycosides amikacin and gentamicin at a concentration of 30 mg/L each. It was evaluated using aminoglycoside-susceptible (*n*=12) and aminoglycoside-resistant (*n*=59) Gram-negative isolates, including 16S rRNA methylase producers (*n*=20). Its sensitivity and specificity of detection were respectively of 95% and 96% for detecting multiple aminoglycoside-resistant methylase producers as well as Gram-negative isolates that produce combinations of aminoglycoside-modifying enzymes, leading to pan-resistance to aminoglycosides. It is only available so far as an home-made version.

Figure 16. 16S rRNA methylase-producing *K. pneumoniae* growing onto the SuperAminoglycoside medium

2.7 Resistance to linezolid

Oxazolidinone antibiotics such as linezolid (LZD) are increasingly used as a consequence of an increased rate of multidrug-resistant Gram-positive pathogens. Considering the wide diffusion of methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis* isolates worldwide, as well as the wide diffusion of vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis*, there is a significant need to rely on the use of LZD to treat infections caused by those multidrug-resistant isolates. Consequently, accurate and rapid identification of LZD-resistant isolates is needed.

The main mechanism of resistance to LZD in Gram-positive bacteria corresponds to a specific mutation (G2576 T) in the 23S rRNA gene, preventing the binding of the drug to its target, i.e. the ribosome [Bi et al., 2017; Gu et al., 2013]. In addition, acquired transferable LZD resistance genes have been reported, namely the *cfr*, *cfr*(B), *cfr*(C), *optrA*, and *poxtA* genes. The *cfr* gene encodes a methylase modifying the C-8 position of A2503 residue in the 23S rRNA methylases, and conferring resistance not only to LZD but also to phenicols, lincosamides, pleuromutilins, and streptogramins (so called $PhLOPS_A$ resistance phenotype). Taking in account the potential clinical threat represented by a diffusion of LZD-resistant strains, our aim was to develop a selective culture medium for screening of LZD-resistant bacteria both among human and animal isolates.

The SuperLinezolid medium was developed for screening resistance to LZD in Gram-positive bacteria (*Staphylococcus* spp., *Enterococcus* spp.) [Nordmann et al., 2019] (Figure 17). It contains LZD at a concentration of 1.5 mg/L. It was evaluated using LZD-susceptible (n = 20) and LZD-resistant (n = 17) Gram-positive isolates. The sensitivity was found to be 82% at 24 h (3 out of 17 isolates being missed), and reached 100% at 48 h. At 48 h, a single LZD-susceptible isolate grew (specificity 95%). By testing stools spiked with LZD-resistant Gram-positive strains, an excellent performance of the medium was observed, with a lowest detection limit ranging from 10^1 to 10^2 CFU/ml. Overall, this medium is accurate for detection of LZD-resistant Gram-positive isolates within 24 h of culture. It has been industrialized under the trade name of CHROMagar LIN-R.

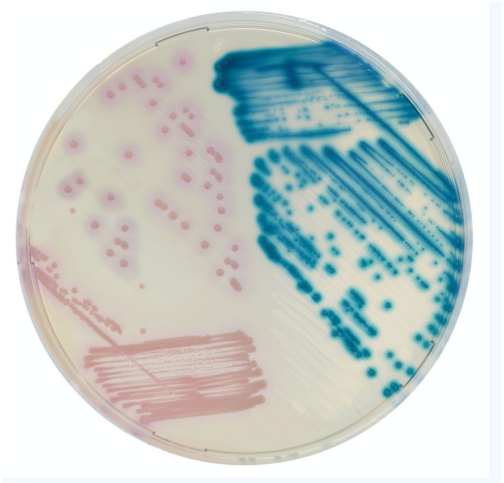


Figure 17. The CHROMagar LinR. Pink colonies correspond to LZD-resistant S. epidermidis while blue colonies correspond to LZD-resistant *Enterococcus* sp.

3- Other test : optimal detection of multidrug-resistant bacteria

We have compared the impact of different approaches, namely non-enrichment, non-selective enrichment, and selective (antibiotic-containing) enrichment steps, for detecting ESBL-producing *Enterobacterales* (ESBL-E), carbapenemase-producing *Enterobacterales* (CPE), polymyxin-resistant Enterobacterales (PMR-E), and vancomycin-resistant enterococci (VRE) from spiked stools (Fournier et al., 2020; Sadek et al., 2020]. The use of a nonselective 18-h enrichment broth culture significantly improved the recovery rate of all types of resistant bacteria after their plating onto selective media. In addition, the detection of ESBL-E, CPE, PMR-E, and VRE was further improved when using an enrichment step using antibiotic-supplemented broths respectively supplemented with cefotaxime (0.1 mg/L), ertapenem (0.1 mg/L), colistin (0.5 mg/L), and vancomycin (1 mg/L). Therefore, we showed here that a screening strategy based on a selective broth enrichment step significantly contributes to an increased rate of detection of multidrug-resistant bacteria, which may be crucial in term of improvement of infection control. In addition, carbapenem-resistant *A. baumannii (*CRAB) and *P. aeruginosa* (CRPA), as well as polymyxin-resistant *A. baumannii* (PMR-AB) and *P. aeruginosa* (PMR-PA), were used to test different enrichment strategies from spiked stools. Three procedures were compared, namely direct inoculation on selective plates and plating after a 24-h enrichment step in tryptic soy broth with or without antibiotics. Selective agar plates were used including CHROMagar-*Pseudomonas* supplemented with meropenem (2 mg/L), and CHROMagar- MDR-*Acinetobacter* agar and CHROMagar COL-APSE plates. Use of enrichment broths significantly enhanced the recovery of CRAB, CRPA, PMR-AB, and PMR-PA. However, supplementing or not the pre-enrichment broth with antibiotics had no impact. The proposed strategy for screening multidrug-resistant non-fermenters is of low cost, is easy to implement, and might be useful for outbreak containment.

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